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Variations in matrix composition and GAG fine structure among scaffolds for cartilage tissue engineering[1]

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Summary

Objective: To compare matrix composition and glycosaminoglycan (GAG) fine structure among five scaffolds commonly used for *in vitro* chondrocyte culture and cartilage tissue engineering.

Design: Bovine articular chondrocytes were seeded into agarose, alginate, collagen I, fibrin and polyglycolic acid (PGA) constructs and cultured for 20 or 40 days. In addition to construct DNA and sulfated GAG (sGAG) contents, the Δ-disaccharide compositions of the chondroitin/dermatan sulfate GAGs were determined for each scaffold group via fluorophore-assisted carbohydrate electrophoresis (FACE).

Results: Significant differences were found in cell proliferation and extracellular matrix accumulation among the five scaffold groups. Significant cell proliferation was observed for all scaffold types but occurred later (20–40 days) in PGA constructs compared to the other groups (0–20 days). By 40 days, agarose constructs had the highest sGAG to DNA ratio, while alginate and collagen I had the lowest levels. Quantitative differences in the Δ-disaccharide composition of the GAGs accumulated in the different scaffolds were also found, with the most striking variations in unsulfated and disulfated Δ-disaccharides. Agarose constructs had the highest fraction of disulfated residues and the lowest fraction of unsulfated residues, with a 6-sulfated/4-sulfated disaccharide ratio most similar to that of native articular cartilage.

Conclusions: The similarities and differences among scaffolds in proteoglycan accumulation and GAG composition suggest that the scaffold material directly or indirectly influences chondrocyte proteoglycan metabolism and may have an influence on the quality of tissue engineered cartilage.

Key words: Cartilage tissue engineering, Chondrocyte, Scaffold, Glycosaminoglycan, FACE.

Introduction

The function of healthy cartilage resides in its ability to provide resistance to compressive, shear and tensile forces that occur during normal joint motion. This ability arises from the unique architecture of the matrix components found in articular cartilage. The fibrillar collagen network is organized and stabilized through molecular cross-links that restrain the swelling pressure exerted by a high concentration of negatively charged proteoglycans. Proteoglycans are known to indirectly influence chondrocyte activity, either through cell–matrix interactions or by binding specific growth factors in the extracellular matrix, thereby modifying their temporal and spatial effects[1]. Many of these biological interactions involve the highly negative glycosaminoglycan (GAG) chains, which are covalently attached to the protein cores of individual proteoglycan molecules[1]. In articular cartilage, the GAGs are mainly chondroitin sulfate (CS) and keratan sulfate chains found on the large, aggregating proteoglycan aggrecan, as well as a small proportion of dermatan sulfate (DS) chains[1]. Aggrecan monomers consist of a protein core to which are attached CS chains containing predominately 4-sulfated disaccharides (ΔDi-4S) or 6-sulfated disaccharides (ΔDi-6S)[2,3]. These negatively charged GAGs produce a swelling pressure through interactions with the ionic interstitial fluid[1], contributing to the compressive stiffness of the tissue and maintaining tensile stress in the type II collagen network. While the collagen network is somewhat metabolically static[4], aggrecan undergoes a distinct turnover process[5,6] in which catabolic cleavage[7,8] and removal of molecules from the extracellular matrix are in balance with synthesis and deposition of new molecules[9,10]. Maintenance of the appropriate structure and concentration of the

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proteoglycans is critical for maintaining healthy cartilage capable of resisting compressive loads.

Alterations in GAG sulfation have been associated with aging and degeneration of articular cartilage. Changes include a decreased keratan sulfate to CS ratio and a decrease in overall GAG chain length[1,11]. The internal disaccharide (Δ-disaccharide) composition of CS chains shifts with age, with an increased ratio of ΔDi-6S/ΔDi-4S and a decrease in unsulfated disaccharides (ΔDi-0S)[1,11]. The non-reducing termini of CS chains from aggrecan purified from fetal to elderly human cartilage contain distinctly different ratios of variably sulfated N-Acetylgalactosamine (Gal NAc) residues which are characteristic of the age of the patient[12], with an age-related increase in 4,6-disulfated residues and a corresponding decrease in 4-sulfated residues. Additionally, a decrease in CS terminal 4,6-disulfated residues and an increase in CS terminal 4-sulfated residues have been described for OA cartilage, perhaps due to a lower propensity to the specific 6-sulfotransferase involved in the terminal 4,6 reaction[2,13–17].

While distinct functional implications of CS sulfation patterns in cartilage have not yet been elucidated, recent work has suggested fundamental biological functions for chondroitin, CS and DS in other systems. These GAGs have been implicated in growth factor and chemokine signaling, play critical roles in the development of the central nervous system, and function as receptors for various pathogens[18–21]. Unsulfated chondroitin has been found to be integral in the morphogenesis and cell division of *C. elegans*[22]. These functions are directly related to the sulfation patterns of the GAG chains.

Understanding the degree and level of influence the CS fine structure plays in the biological activity of chondrocytes may provide a more in depth perspective on not only the state of native cartilage (i.e., osteoarthritic, injured, healthy) but also the development of engineered cartilages. A key component of most tissue engineering approaches to regenerating articular cartilage is the choice of a scaffold material. The three-dimensional scaffold provides support for chondrocytes to proliferate and maintain their differentiated function[23–26]. In the case of undifferentiated cell populations (e.g., bone marrow stromal cells, mesenchymal stem cells), the choice of scaffold may influence the differentiation and phenotypic stability of the cell populations. Scaffolds commonly used for the tissue engineering of cartilage include degradable synthetic polymers (e.g., polyglycolic acid (PGA)[27,28], polylactic acid[29]), fibrillar protein gels (e.g., collagen[30], fibrin[31,32]), and polysaccharide gels (e.g., agarose[33,34], alginate[35,36]). Matrix formation in these systems is typically quantified by measures of gross biochemical composition such as the total sulfated GAG (sGAG) content. While these gross measures are useful, more definitive molecular information is required to fully understand the biochemical characteristics of tissue engineered cartilage matrices. Therefore, this study examined the nature of the proteoglycan/GAG matrices synthesized by chondrocytes seeded in different scaffold materials. In addition to differences in total GAG content, fluorophore-assisted carbohydrate electrophoresis (FACE) revealed differences in CS/DS GAG fine structure among different scaffold materials.

Methods

MATERIALS

Bovine stifle joints were from Research 87 (Marlborough, MA). Collagenase type 2 was from Worthington Biochemical (Lakewood, NJ). Fetal bovine serum (FBS), high glucose Dulbecco's Modified Eagle's Medium (DMEM), phosphate buffered saline (PBS), non-essential amino acids (NEAA), sodium pyruvate, L-glutamine and gentamicin sulfate were from Gibco BRL (Rockville, MD). Low gelling temperature agarose (type VII), ascorbate, 1,9-dimethyl-methylene blue (DMMB), calf thymus DNA, Hoechst 33258 dye and Safranin-O were from Sigma (St. Louis, MO). Rat-tail collagen type I was from Collaborative Biomedical Products (Bedford, MA). 6-Aminocaproic acid was from Acros Organics (Fairlawn, NJ). PGA felts were from Albany International (Mansfield, MA). Sodium alginate (UP LVG) was from Pronova Biomedical (Oslo, Norway). Bovine fibrinogen and bovine thrombin were from ICN Biomedicals (Irvine, CA). 2-Aminoacridone hydrochloride was from Molecular Probes (Eugene, OR). Mono composition gels and mono gel running buffer were from Glyko (Novato, CA). Disaccharide standards, chondroitinase ABC, chondroitinase AC and hyaluronidase were from Calbiochem (La Jolla, CA). Imaging was done with an EDAS 120 imaging system from Eastman Kodak (New Haven, CT).

CONSTRUCT ASSEMBLY AND CELL CULTURE

Bovine articular chondrocytes were isolated from the stifle joints of two immature (<5 weeks) donor animals. Tissue was excised from the patellofemoral grooves, minced and digested in DMEM with 0.2% collagenase for 24 h in a 37°C, 5% CO_2 incubator. After centrifugation at 160 × *g*, cells were washed twice with Ca^{2+}, Mg^{2+}-free PBS and counted with a Coulter counter. Donor cells were kept separate and parallel duplicate experiments were run. Non-digested samples of cartilage were maintained as controls during the course of the experiment. Cells were seeded at a final density of 15×10^6 cells/ml in 1.5% agarose, 0.75% sodium alginate, 2 mg/ml type I collagen (rat tail), and 50 mg/ml fibrin. Cells were also seeded on PGA felt at a target density of 15×10^6 cells/ml, but a low seeding efficiency resulted in an actual initial cell density of approximately 4×10^6 cell/ml. It should be noted that seeding density may affect chondrocyte matrix synthesis and viability, perhaps due to diffusional limitations at high cell densities[34,37]. All constructs except PGA were cast in 12 mm diameter by 3 mm deep cylindrical wells in custom machined polycarbonate molds that were autoclave sterilized prior to gel casting. Agarose constructs were assembled by autoclaving 3% LMP agarose in 1× Ca^{2+}, Mg^{2+}-free PBS and then cooling the solution to 42°C. An equal volume of cells suspended at 30×10^6 cells/ml in 2× DMEM and 20% FBS was added, and the solution was cooled in the molds. Alginate constructs were similarly assembled by autoclaving 1.5% alginic acid in 1× Ca^{2+}, Mg^{2+}-free PBS to solubilize and sterilize, cooling to 37°C, adding 30×10^6 cells/ml in Ca^{2+}, Mg^{2+}-free PBS and polymerizing the gels in the molds with 102 mM $CaCl_2$. Collagen constructs were assembled by suspending 15×10^6 cell/ml in a solution containing 10% FBS, 0.5× DMEM and 2 mg/ml acid solubilized rat-tail collagen type I and titrating the solution to neutral pH with 0.1 M NaOH. Constructs were allowed to polymerize in a 37°C, 5% CO_2 incubator for 30 min before transfer to 24-well plates with 2 ml of media. Fibrin constructs were assembled by suspending cells in a solution of bovine fibrinogen, FBS, 6-aminocaproic acid, and DMEM. Bovine thrombin was dissolved in 40 mM $CaCl_2$, and 100 μl of the thrombin solution was placed into each well of a polycarbonate mold, followed by 200 μl of the cell/fibrinogen mixture. Final

concentrations in the constructs were 50 mg/ml fibrinogen, 50 U/ml thrombin, 10% FBS, 2 mg/ml 6-aminocaproic acid, and 15×10^6 cells/ml. Constructs were allowed to polymerize in a 37°C, 5% CO_2 incubator for 30 min. PGA felts (45 mg/cm^3 polymer density; 12–15 μm fiber thickness; 10 mm diameter by 2 mm thickness) were prewetted overnight in high glucose DMEM containing 10% FBS. PGA felts were then seeded by repeatedly pipetting 1 ml of cell suspension (1.2×10^6 cells/ml) onto each felt and cultured on a shaker plate at 300 rpm for 24 h. Additionally, native cartilage samples from the same joints were maintained in identical culture conditions for the comparison of Δ-disaccharide composition.

Constructs and native cartilage samples ($n = 6$ per group per endpoint) were cultured in 24-well plates with 2 ml of DMEM containing 10% FBS, 0.1 mM NEAA, 4 mM L-glutamine, 5 μg/ml gentamicin sulfate and 50 μg/ml ascorbic acid. For the PGA felts, the culture dish wells had been pre-coated with a thin layer of 1% agarose. As in previous studies on dorsal root ganglia[38], smooth muscle cells[39] and chondrocytes[40,41], 6-aminocaproic acid (2 mg/ml) was added to the media of fibrin constructs to inhibit proteolytic degradation. Media were changed every 2 days, with samples retained for analysis of the sGAG released from the constructs. After 20 and 40 days, constructs were weighed, lyophilized, reweighed, and digested in 1 ml of 100 mM ammonium acetate buffer with 250 μg/ml Proteinase K at 60°C for 24 h. Portions of each digest were assayed for total DNA using the Hoechst 33258 assay with calf thymus DNA as a standard[42] and sGAG using the DMMB assay[43,44] with shark cartilage CS as a standard.

HISTOLOGICAL SAMPLE PROCESSING AND CHARACTERIZATION

Additional samples were rinsed in PBS, transferred to 10% neutral-buffered formalin for 48 h, and then stored in 70% ethanol ($n = 2$ per group per endpoint) to reduce the solubility of the GAG side chains. Samples were subsequently embedded in paraffin and sectioned at 5 μm. Sections were stained with Safranin-O to visualize sGAG distribution[45].

Δ-DISACCHARIDE ANALYSIS

The CS and DS (CS/DS) Δ-disaccharide compositions of deposited GAGs were quantified for all 20- and 40-day constructs using FACE[46–50]. GAGs were ethanol precipitated (final concentration of 75% v/v) from the Proteinase K digests and aliquots from each construct containing 5 μg of sGAG (as determined by the DMMB dye assay) were further enzymatically processed with 15 mU of chondroitinase ABC and 100 mU hyaluronidase in 100 μL of 50 mM ammonium acetate buffer at 37°C for 16 h. Buffer was evaporated and digestion products were fluorescently labeled for 16 h at 37°C with 15 μL of 0.1 M 2-aminoacridone and 1.0 M sodium cyanoborohydride. After fluorescent labeling, 15 μL of glycerol was added to each sample. Portions (5 μL) of these samples were electrophoretically separated using monosaccharide FACE gels. Fluorescent bands were visualized at 300 nm illumination using a Kodak EDAS 120 gel imaging system. Band intensities were fit with a Gaussian model and converted to pmol amounts using a standard ladder containing a range of concentrations of purified Δ-disaccharides (25–250 pmol) for product identification and quantification (50 pmol of disulfated disaccharides (ΔDi-4,6S), 75 pmol hyaluronic acid, 100 pmol of ΔDi-6S, 150 pmol of ΔDi-4S, and 200 pmol of ΔDi-0S). Relative Δ-disaccharide composition was expressed as a percent of total detected internal CS/DS Δ-disaccharides for ΔDi-0S, ΔDi-4S, ΔDi-6S and ΔDi-4,6S and as the ratio of ΔDi-6S/ΔDi-4S. For comparison, native articular cartilage samples that had been cultured for comparable periods were also analyzed via FACE.

STATISTICAL ANALYSIS

As no substantial differences in proliferation or matrix production were found between the donors, data were analyzed with two factor (scaffold, time point) general linear models with Tukey's test for pair-wise comparisons. Differences were considered significant at $P < 0.05$. All results are reported for pooled data from the two donors.

Results

HISTOLOGY

For a given construct type, no differences were noted in the organization of cells or sGAG matrix between histological samples from the 20- and 40-day time points. Cells in all scaffolds displayed predominantly a rounded morphology [Fig. 1(B–F)] similar to chondrocytes in their native matrix [Fig. 1(A)]. Agarose constructs [Fig. 1(B)] included isolated cells and multiple cell clusters surrounded by a pericellular matrix dense in sGAG, while the other constructs contained primarily isolated cells or small cell clusters. The intensity of pericellular matrix staining in the four other scaffold materials was either considerably less intense (alginate, collagen and PGA) or less extensive (fibrin) than that in agarose. Alginate constructs [Fig. 1(C)] also displayed intense pericellular matrix staining, with a relatively high background level of staining due to the negatively charged alginate. Collagen constructs [Fig. 1(D)] contained uniformly distributed cells and diffuse matrix staining. In contrast, fibrin constructs [Fig. 1(E)] displayed a very immediate staining of sGAG surrounding either single cells or pairs of cells. PGA constructs [Fig. 1(F)] contained islands with relatively intense matrix staining and numerous cells surrounded by regions apparently devoid of cells. Articular cartilage, alginate and fibrin constructs were surrounded by a layer with little sGAG staining and cells with a more flattened morphology (images not shown).

DNA CONTENT

The relative changes in DNA content from initial seeding to days 20 and 40 indicated differential increases in the cellular contents among scaffold types (Fig. 2). The increases in DNA contents of agarose and collagen constructs were not significantly different at either time point ($P > 0.8$) and reached approximately 3.5 times the initial seeding density. Agarose constructs had significantly greater increases in DNA content than alginate, fibrin or PGA constructs at both day 20 ($P \leq 0.0002$) and day 40 ($P \leq 0.028$). Collagen constructs had significantly greater increases in DNA content than fibrin and PGA constructs at day 20 ($P \leq 0.0016$) and day 40 ($P \leq 0.0001$), and significantly greater increases than alginate constructs at day 40 ($P < 0.0001$) but not day 20 ($P = 0.061$). There were no significant changes in DNA content between 20 and 40 days for any constructs except for PGA ($P < 0.0001$). PGA constructs exhibited a small (25%) increase in DNA content by day 20, followed by a substantial

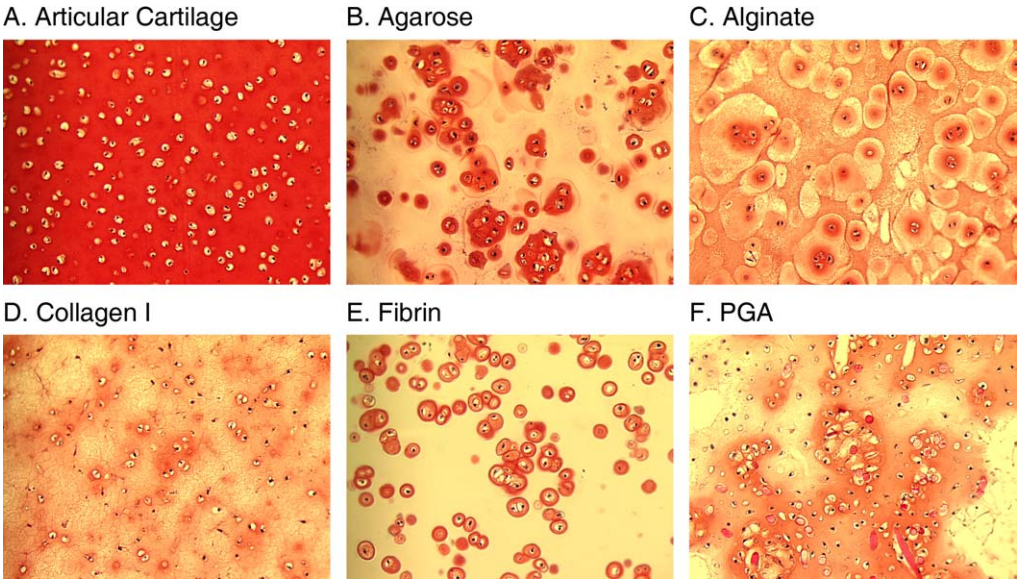

Fig. 1. Representative interior sections stained with Safranin-O from (A) articular cartilage and (B–F) constructs cultured for 20 days.

(109%, $P < 0.0001$) increase in DNA content between days 20 and 40, resulting in a net increase comparable to that seen in the other construct types. At day 40, alginate, fibrin and PGA constructs had reached approximately 2.5 times the original seeding density, with no statistically significant differences among these scaffolds.

sGAG PRODUCTION

The sGAG/DNA results indicated that constructs from all scaffold groups accumulated extracellular matrix over the 40-day culture period (Fig. 3). Between days 20 and 40, agarose constructs exhibited a continued increase in sGAG/DNA ($P < 0.0001$) while alginate, collagen, and fibrin constructs exhibited no significant change in sGAG/DNA. PGA constructs had significantly lower sGAG/DNA at day 40 as compared to day 20 ($P < 0.0001$), which can be attributed to the dramatic increase in DNA content over that period. The patterns of matrix accumulation and cell content changes resulted in differences in sGAG/DNA among scaffolds at each time point. At day 20, PGA constructs had significantly higher sGAG/DNA than all other groups ($P < 0.0001$), while alginate constructs had significantly lower sGAG/DNA than all groups ($P \leq 0.0023$) except collagen constructs ($P = 0.45$). At day 40, agarose constructs had significantly higher sGAG/DNA than all other groups ($P < 0.0001$), while collagen and alginate constructs had significantly lower sGAG/DNA ($P \leq 0.0003$ and $P < 0.0001$, respectively) than all other groups.

The sGAG detected in the media indicated that sGAG was released from all constructs through the 40-day culture period (Fig. 4) Overall, the average sGAG release rate was lowest for alginate constructs and comparable for agarose, collagen and PGA constructs. The kinetics of sGAG release were similar for agarose, alginate, collagen and fibrin, with

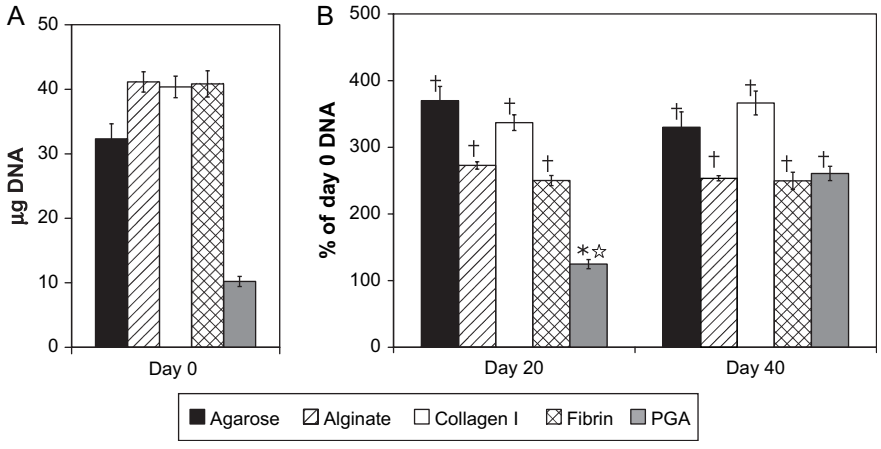

Fig. 2. Construct DNA contents. (A) DNA contents at day 0. PGA constructs had substantially fewer cells due to a low seeding density. (B) DNA contents relative to day 0 levels for each scaffold type after 20 and 40 days of culture. Asterisks indicate $P < 0.05$ vs all other scaffolds at the same time point, daggers indicate $P < 0.05$ vs some other scaffolds at the same time point, and stars at day 20 indicate $P < 0.05$ vs the day 40 value for the same scaffold. Individual differences are discussed in the text (mean $\pm$ S.E.M., $N = 12$).

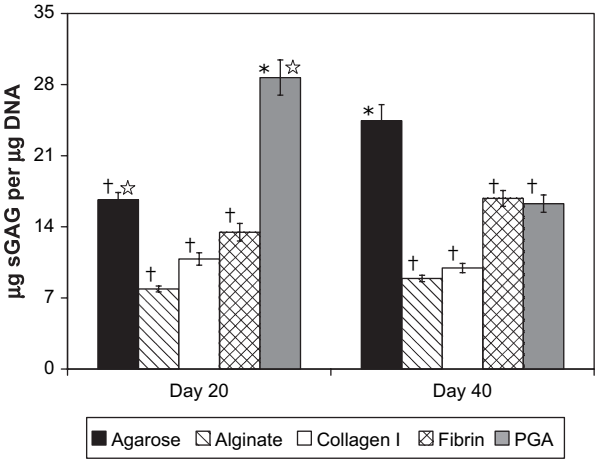

Fig. 3. Construct sGAG content normalized to DNA content after 20 and 40 days of culture. Asterisks indicate $P < 0.05$ vs all other scaffolds at the same time point, daggers indicate $P < 0.05$ vs some other scaffolds at the same time point, and stars at day 20 indicate $P < 0.05$ vs the day 40 value for the same scaffold. Individual differences are discussed in the text (mean ± S.E.M., $N = 12$).

increasing release rates through day 30, followed by a significant (except for agarose) drop in sGAG release on days 31−40. In contrast, the sGAG release rate for PGA constructs was highest on days 1−10 and decreased steadily though day 40.

Δ-DISACCHARIDE COMPOSITION

As in native articular cartilage, ΔDi-4S and ΔDi-6S comprised the majority (~80%) of CS/DS-derived Δ-disaccharides (Table I), although the fraction of each Δ-disaccharide varied somewhat. Overall, the fraction of ΔDi-4S was lower at day 20 than at day 40 ($P = 0.0025$), although the pair-wise comparisons did not indicate a significant increase for any individual scaffold. The fraction of ΔDi-4S was higher for agarose than for all other scaffolds ($P \leq 0.0002$). This pair-wise difference was significant at day 20 versus all scaffolds ($P < 0.0001$) except collagen ($P = 0.053$) and was significant at day 40 vs fibrin ($P = 0.031$) and PGA ($P = 0.034$) but not collagen ($P = 0.055$) or alginate ($P = 0.25$). Other than a higher fraction for collagen compared to PGA at day 20 ($P = 0.014$), there were no other significant differences in ΔDi-4S among scaffolds at either time point. The fraction of ΔDi-6S was not significantly different between day 20 and day 40 ($P > 0.19$), but did vary among scaffolds ($P < 0.0001$). Among the construct groups, the fraction of ΔDi-6S was smaller for agarose than for all other scaffolds ($P \leq 0.032$) and was smaller for collagen than for alginate ($P = 0.0017$) and PGA ($P = 0.021$). The ratio of ΔDi-6S/ΔDi-4S in the constructs was generally somewhat higher than that in native tissue explants (Fig. 5). The ratio of ΔDi-6S/ΔDi-4S was slightly higher at day 20 than at day 40 ($P = 0.009$), was lower for agarose than for all other scaffolds ($P \leq 0.0019$), and was lower for collagen than for alginate ($P = 0.007$) and PGA ($P = 0.0075$).

Overall, the fraction of ΔDi-4,6S was significantly higher at day 20 than at day 40 ($P = 0.0017$), but other than a slightly higher level for fibrin constructs at day 40 ($P = 0.078$) there were no significant differences in the fraction of ΔDi-0S between days 20 and 40. There were significant differences among scaffold types in the fractions of both ΔDi-0S and ΔDi-4,6S. Alginate constructs had a lower fraction of ΔDi-0S than all other scaffold groups ($P < 0.0001$) and a higher fraction of ΔDi-4,6S than all other scaffold groups ($P < 0.0001$). In contrast, collagen constructs had a higher fraction of ΔDi-0S than all other scaffold groups ($P \leq 0.023$) and a lower fraction of ΔDi-4,6S than alginate, fibrin or PGA ($P \leq 0.0001$) constructs. Other than a significantly higher level of ΔDi-0S for agarose than for fibrin at day 20 ($P = 0.0084$), there were no other significant differences among scaffold groups in the fractions of ΔDi-0S or ΔDi-4,6S.

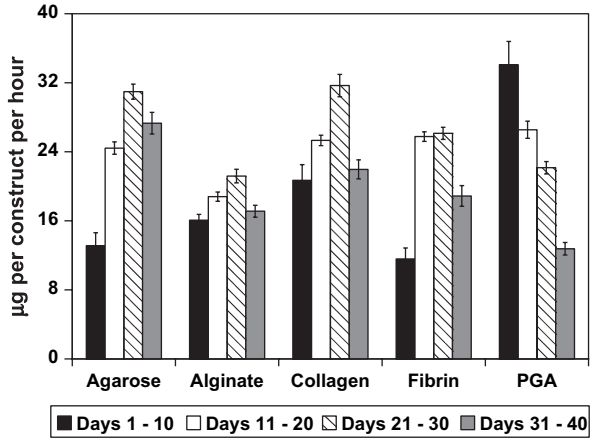

Fig. 4. Average sGAG release rates during four 10-day intervals (mean ± S.E.M., $N = 20$).

Discussion

While a wide variety of biomaterials have been proposed for use in cartilage tissue engineering, few studies have quantitatively compared details of chondrocyte activity and extracellular matrix production in different scaffold materials. This study found significant differences in cell proliferation and extracellular matrix accumulation among five scaffolds commonly used for chondrocyte *in vitro* culture studies and cartilage tissue engineering. Over the first 20 days, PGA constructs had the lowest increase in cell content but the greatest accumulation of sGAG per cell and the greatest rate of sGAG release to the media. Between day 20 and day 40, the sGAG/DNA of the other constructs was fairly stable while that of PGA constructs decreased substantially due to the substantial increase in cell proliferation in PGA scaffolds. Chondrocytes in agarose synthesized the most sGAG throughout the study, resulting in a substantially higher sGAG/DNA than all other scaffolds at day 40. Distinct differences between scaffolds were also noted in the distributions of cells and matrix. The differences in the distribution of the chondrocytes in the various scaffolds may be due to inherent differences in the preparation required for the various scaffolds or in how the cells interact with particular scaffolds after seeding. Other possible reasons for the cell clustering include the migration of cells into colonies or localized cell death.

Furthermore, this study revealed substantial quantitative differences in the Δ-disaccharide composition of the proteoglycans accumulated in the different scaffolds.

Overall, the internal Δ-disaccharide profiles of GAGs in alginate constructs least resembled that of native articular cartilage, whereas the profile of agarose constructs most closely resembled that of articular cartilage. The primary notable difference in the levels of the monosulfated ΔDi-4S and ΔDi-6S was for agarose constructs, which had a higher fraction of ΔDi-4S, lower fraction of ΔDi-6S, and lower ΔDi-6S/ΔDi-4S than all other scaffold groups. More striking differences were seen in the levels of ΔDi-0S and ΔDi-4,6S. Alginate constructs had a lower fraction of unsulfated residues and a higher fraction of disulfated residues than other groups. Conversely, collagen constructs had the highest fraction of unsulfated residues and the lowest fraction of disulfated residues. These patterns suggest that some aspects of the construct environment influence a tradeoff between incorporation of ΔDi-0S and ΔDi-4,6S into newly synthesized proteoglycans. Further studies would be necessary to identify the causes of the observed differences in sulfation patterns (e.g., altered sulfotransferase activity, altered ratios of proteoglycan species, etc.). Interestingly, the influence of mechanical stimulation on GAG disaccharide composition also appears to depend on the cellular environment. Cyclic compression of cartilage tissue explants had no effect on the internal disaccharide composition of newly synthesized GAGs[51], while cyclic compression of chondrocytes in fibrin constructs significantly decreased the proportion of ΔDi-0S[40]. Further exploration of variations between scaffolds in response to mechanical stimulation will be important in the development of mechanically functional engineered cartilage constructs.

The similarities and differences among scaffolds in proteoglycan accumulation and GAG composition were consistent for both donor animals at both time points, suggesting that the scaffold material directly or indirectly influences chondrocyte proteoglycan metabolism. Prior to the deposition of any extracellular matrix, chondrocyte interactions with the scaffold would vary substantially depending on the type of scaffold. Direct chondrocyte binding to protein scaffolds such as collagen or fibrin via integrins or other cell surface receptors may influence a variety of cellular functions[52–54]. In contrast, chondrocytes would initially be isolated from interactions with the scaffold material when suspended in the agarose and alginate hydrogels. In PGA scaffolds, chondrocytes initially adhere to and spread on serum proteins absorbed onto the polymer mesh, resulting in an initial state somewhat similar to monolayer culture[55,56].

In each of these systems, chondrocytes would eventually interact primarily with the new extracellular matrix produced by the cells, but the accumulation of that matrix could be influenced by the physical properties of the scaffold[57]. For example, the highly permeable, macroscopically porous PGA scaffolds initially provide little resistance to diffusion of secreted proteins and proteoglycans, allowing much of the newly synthesized material to escape the construct[56,57]. Conversely, scaffolds such as alginate and agarose provide substantial resistance to diffusion of large molecules, resulting in more concentrated pericellular accumulation of secreted matrix components[57]. Additionally, the biophysical environment of the cell may vary between scaffolds. For example, the agarose gel provides an electrochemically neutral environment, while the negatively charged alginate may affect both the ionic environment and the interstitial pH, potentially influencing cell behavior and the ability of the local environment to sequester important growth factors, cytokines and chemokines.

While the functional implications of altered Δ-disaccharide sulfation patterns in native or engineered cartilage are unknown, there is evidence of structure–function relationships in other tissues and with other sGAGs. Oversulfation of CS/DS chains containing GlcAβ1-3GalNAc(4,6-O-disulfate) or IdoAα1-3GalNAc(4,6-O-disulfate) in the large aggregating proteoglycan versican affects its ability to bind to L- and P-selectin and chemokines[21]. Additionally, any amount of sulfation has been shown to inhibit the ability of versican to interact with CD44[21]. It has been shown that oversulfated CS chains play a role in the development of the brain. Specifically, CS chains with oversulfated structures are involved in neuronal adhesion, migration and neuritogenesis[58]. Thus, the possibility exists that variations in the sulfation of the CS chains in cartilage may alter the binding of nutrients, chemokines and growth factors, thereby influencing chondrocyte migration, differentiation, and matrix production.

With advances in tissue engineering, it has become apparent that more sophisticated methods of evaluating engineered cartilage constructs need to be employed. In this study, articular chondrocytes seeded into different scaffolds exhibited differences in proliferation, the quantity

Table I

GAG Δ-disaccharide contents derived from CS and DS after 20 and 40 days of culture. Levels for articular cartilage cultured for comparable periods are included for visual comparison. Values are expressed as percentages of total Δ-disaccharide measured. Asterisks indicate $P < 0.05$ versus all other scaffolds at the same time point and daggers indicate $P < 0.05$ versus some other scaffolds at the same time point (mean ± S.E.M., $N = 12$)

	ΔDi-0S	ΔDi-6S	ΔDi-4S	ΔDi-4,6S
20 Days				
Agarose	14.27 ± 0.31†	38.65 ± 0.46*	40.62 ± 0.61†	6.46 ± 0.12†
Alginate	7.43 ± 0.19*	44.49 ± 0.83†	34.99 ± 0.89†	13.08 ± 0.29*
Collagen I	16.89 ± 0.30*	40.82 ± 0.32†	37.51 ± 0.33†	4.78 ± 0.17†
Fibrin	11.86 ± 0.52†	42.93 ± 1.18†	35.46 ± 0.93†	9.75 ± 1.51†
PGA	13.51 ± 0.42†	42.59 ± 0.46†	33.99 ± 0.29†	9.91 ± 0.41†
Articular cartilage	12.56 ± 0.87	37.12 ± 0.51	45.10 ± 1.32	5.21 ± 0.26
40 Days				
Agarose	14.91 ± 0.35†	38.20 ± 0.65*	40.26 ± 0.39†	6.63 ± 0.43†
Alginate	8.09 ± 0.31*	42.50 ± 1.60†	37.78 ± 1.70	11.63 ± 0.54*
Collagen I	17.69 ± 0.39*	40.41 ± 0.38†	37.17 ± 0.58	4.73 ± 0.23†
Fibrin	14.27 ± 0.70†	42.02 ± 0.94†	36.97 ± 0.97†	6.73 ± 1.65†
PGA	12.90 ± 0.50†	43.25 ± 0.48†	37.02 ± 0.49†	6.82 ± 0.26†
Articular cartilage	14.69 ± 0.76	36.53 ± 0.66	42.91 ± 0.99	5.87 ± 0.24

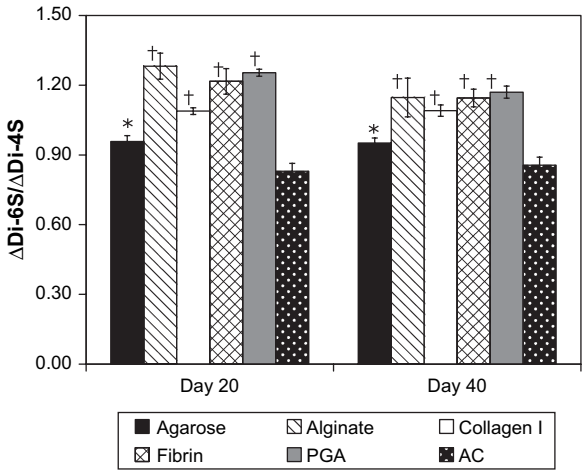

Fig. 5. Construct ΔDi-6S/ΔDi-4S ratios after 20 and 40 days of culture. Levels for articular cartilage cultured for comparable periods are included for visual comparison. Asterisks indicate $P < 0.05$ vs all other scaffolds at the same time point and daggers indicate $P < 0.05$ vs some other scaffolds at the same time point. Individual differences are discussed in the text (mean ± S.E.M., $N = 12$).

of matrix accumulated and the fine structure of the newly synthesized GAGs. It should be noted that many evolving approaches to cartilage tissue engineering rely on either passage expanded chondrocytes or chemically manipulated progenitor cells, either of which would be expected to continue differentiating towards a chondrocytic phenotype within the construct environment. In such cases where relatively rapid changes in phenotype are expected, the influence of the scaffold environment on cell behavior and matrix composition may be more pronounced. An appreciation of the details of the extracellular matrix structure in engineered tissues may be important in understanding the relative maturity and health of the engineered tissue and the degree to which engineered implants may differ from and integrate with the surrounding native tissues.

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